THE THIOBACILLI[1, 2]

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MICROBIAL TRANSFORMATIONS OF SULFUR COMPOUNDS

"Une synthèse complète de la matière organique par l'action d'êtres vivants peut s'accomplir sur notre planète indépendamment des rayons solaires."—Sergei Winogradsky (1).

Winogradsky's observation, 70 years ago, that H_2S could serve as electron donor in the respiration of *Beggiatoa* sp. was the first in his series of studies which culminated in the discovery of the chemoautotrophic mode of life (2). It is ironic that, although other autotrophic sulfur bacteria are known today, *Beggiatoa* itself has not yet conclusively been shown to be chemoautotrophic. The experiments of Cataldi suggest that at least some strains of *Beggiatoa* are heterotrophic (3). Nevertheless, Winogradsky was correct in envisioning an ecological niche in which a microbe derives all energy from the oxi-

[1] The authors wish to dedicate this review to the memory of A. J. Kluyver whose formulation of the comparative biochemistry of hydrogen transfer has contributed greatly to our understanding of autotrophic metabolism.

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dation of sulfur and all carbon from carbon dioxide. Furthermore, Winogradsky's studies on *Beggiatoa* and other sulfur bacteria stimulated Nathansohn to attempt the cultivation of some large colorless marine microbes which he took to be sulfur bacteria (4). To his disappointment Nathansohn failed to cultivate any of Winogradsky's organisms in sea water supplemented with sodium thiosulfate. Instead, his medium swarmed with a minute polarly flagellated member of the *Eubacteriales*, an organism which Nathansohn did not name but which Beijerinck two years later called *Thiobacillus thioparus* (5). Nathansohn quickly established that this newly found bacterium grew at the expense of the added thiosulfate, that the amount of growth was strictly proportional to the amount of sulfate formed, and that all carbon was derived from carbon dioxide. The organism therefore conformed to Winogradsky's definition of a chemoautotroph.

The ability to oxidize reduced sulfur compounds is not limited to autotrophic bacteria. The work of Guittonneau has revealed that the oxidation of reduced sulfur compounds in nature can be brought about by a variety of heterotrophic microorganisms (6, 7). Guittonneau studied the fate of sulfur which was used to dust vineyards and found that thiosulfate and polythionates arose in the soil at the expense of molecular sulfur, to be ultimately oxidized to sulfate. He isolated a number of common soil organisms which were able to carry out some of these oxidative steps and which in conjunction oxidized sulfur to sulfate. Heterotrophic bacteria which oxidize thiosulfate have also been isolated by Starkey (8) and Sijderius (9). It is likely that in most soils the oxidation of sulfur compounds mediated by members of the genus *Thiobacillus* is insignificant compared to that carried out by heterotrophic organisms.

The oxidation of reduced sulfur compounds by chemosynthetic, photosynthetic, and various heterotrophic organisms gives rise to sulfate. This oxidation is balanced in nature by the biological reduction of sulfate to sulfide. Sulfate may serve as a sulfur source for plants and for most microorganisms. The largest single agency for sulfate reduction is the sulfate reducing bacterium, *Desulfovibrio desulfuricans* and its allies, for which sulfate is the terminal respiratory electron acceptor. The production of H_2S by this bacterium is of sufficient magnitude to give rise to geological deposits of sulfides and sulfur (10–14).

SYSTEMATIC DESCRIPTION OF THE THIOBACILLI

The genus *Thiobacillus*, to which this review is limited, comprises a small number of closely related species. The members of this genus are gram negative, nonsporulating rods, measuring 0.5 by 1–3 μ, which, except for the nonmotile *Thiobacillus novellus*, are polarly flagellated. They may thus be a specialized group of pseudomonads. All members of the genus use reduced sulfur compounds such as thiosulfate as electron donors and carbon dioxide as carbon source. With the exception of *T. novellus*, they are unable to utilize any organic compounds and are therefore strict autotrophs in Winogradsky's sense of the word. Despite their inability to grow at the expense of organic substrates, which remains a major puzzle in the study of their physiology, it can be shown that their rate of respiration is influenced by the addition of succinate or several other members of the tricarboxylic acid cycle (15, 16) and that added glucose will gradually disappear from a culture growing on sulfur (17). It has also been shown by Rittenberg and Grady that the irradiation of *Thiobacillus thiooxidans* with ultraviolet light can give rise to mutants which require one or more vitamins (18).

Thiobacillus thioparus. The type species, *T. thioparus*, is the organism which Nathansohn studied in 1902 (4) and which Beijerinck reisolated and described in 1904 (5). It is a strictly aerobic bacterium which grows rapidly in a mineral medium containing thiosulfate, and frequently deposits large amounts of molecular sulfur. These sulfur deposits are especially conspicuous on agar plates and give the colonies a milky white to canary yellow appearance. Some strains, particularly those isolated from fresh water, produce a ropy mucoid slime in which the bacteria are embedded and hence appear nonmotile. Marine isolates of *T. thioparus* are generally vigorously motile and grow more rapidly than fresh water isolates. Concentrated masses of *T. thioparus* appear bright orange to red as a result of their high content of a cytochrome *c*. The difference spectrum of this cytochrome shows absorption maxima at 420, 522, and 552 mμ (unpublished observation). The organism grows most rapidly near pH 7 at 30 C.

Thiobacillus denitrificans. T. denitrificans is

closely related to *T. thioparus*, differing from the latter only in its ability to use nitrate as a terminal respiratory electron acceptor under anaerobic conditions. This sulfur bacterium was first observed in 1904 by Beijerinck (5). It is improbable that he obtained a pure culture since he reported that the organism was a facultative autotroph and became irreversibly heterotrophic on subculture in organic media. A few years later Lieske (19) and Gehring (20) described *T. denitrificans* as an obligate autotroph. Their reports have been confirmed by contemporary studies (21). Like other denitrifying bacteria, as for instance *Pseudomonas stutzeri*, *T. denitrificans* can also grow under air in the absence of nitrate but rapidly loses its denitrifying ability on aerobic subculture. *T. denitrificans* cultivated aerobically therefore becomes indistinguishable from *T. thioparus*. A similar relationship may exist between the facultatively autotrophic denitrifying thiobacillus observed by Tyulpanova-Mosevich (22) and Starkey's *T. novellus* (8).

Thiobacillus thiooxidans. In 1914 Lockett reported the occurrence in sewage of a sulfur oxidizing organism which fixed carbon dioxide and turned the medium "acid to methyl orange" (23). Waksman and Joffe seven years later isolated from soil a sulfur oxidizing bacterium, very likely identical with Lockett's, which they named *T. thiooxidans* (24). In contrast to *T. thioparus* and *T. denitrificans*, both of which prefer a neutral environment, *T. thiooxidans* grows best below pH 5 and has been known to produce a negative pH (25). Aside from its acid habitat, this bacterium is primarily distinguished by its ability to oxidize elemental sulfur at a rate comparable to its oxidation of thiosulfate, in contrast to *T. thioparus* and *T. denitrificans* which oxidize elemental sulfur more slowly.

Thiobacillus ferrooxidans and Ferrobacillus ferrooxidans. An iron oxidizing sulfur bacterium has been described by Colmer *et al.* (26, 27) and classified as *T. ferrooxidans*. It was assigned to the genus *Thiobacillus* because it is an obligate autotroph, can use thiosulfate as an electron donor, and is morphologically identical with *T. thiooxidans* and *T. thioparus*. Although *T. ferrooxidans* is favored by the same pH range as *T. thiooxidans*, it can be differentiated from the latter by its inability to oxidize elemental sulfur rapidly. *T. ferrooxidans* differs from all other known thiobacilli in the ability, from which it derives its name, to use Fe^{++} ions instead of thiosulfate as electron donors. Repeated subculture of *T. ferrooxidans* on an iron-containing medium results in loss of its ability to oxidize thiosulfate, although thiosulfate-grown cells are still capable of oxidizing ferrous salts (K. L. Temple, private communication). The validity of the species *T. ferrooxidans* has been questioned by Leathen *et al.* (28, 29), who have failed to find a single bacterium capable of oxidizing both thiosulfate and ferrous iron. Instead these authors believe that the acidification of bituminous coal mine effluents can be ascribed to two bacteria, *T. thiooxidans* and *Ferrobacillus ferrooxidans*. The obligately autotrophic *F. ferrooxidans* is morphologically indistinguishable from *T. thiooxidans*, its pH optimum is also near 3.5, but ferrous iron is its sole oxidizable substrate (30). Although the iron oxidizing bacterium may therefore not be a member of the genus *Thiobacillus* it appears to be closely related to it. The discovery of *F. ferrooxidans* has vindicated Winogradsky's belief that the oxidation of Fe^{++} ions to Fe^{+++} ions could serve to support the growth of a CO_2 fixing microbe. Winogradsky had long ago claimed that a number of microorganisms, such as *Leptothrix ochracea*, respired by oxidizing Fe^{++} ions in the medium and assimilated CO_2 as their only carbon source (31–33). It now appears that Winogradsky's original iron bacteria are heterotrophs which under suitable environmental conditions may embed themselves in deposits of ferric hydroxides (34, 35).

Thiobacillus thiocyanoxidans. Happold and Key found that ammonium thiocyanate, which is formed as a waste product in the manufacture of illuminating gas, undergoes microbial oxidation (36). A re-examination of the flora responsible for this oxidation led to the discovery of an obligately autotrophic bacterium, *Bacterium thiocyanoxidans*, for which thiocyanate can serve as a source of energy, nitrogen, and carbon (37). Since this organism can also oxidize sulfide and thiosulfate and agrees in morphology with the motile thiobacilli, Happold *et al.* renamed it *Thiobacillus thiocyanoxidans* (38). The physiology of *T. thiocyanoxidans* has been studied by Youatt (39). She concluded that thiocyanate is hydrolyzed to cyanate and sulfide, followed by an oxidation of sulfide to sulfate. Cyanate is hydrolyzed to ammonia and carbon dioxide, part of the latter being assimilated during growth. Cells grown on thiosulfate oxidize thiocyanate

only slowly, while thiocyanate grown cells rapidly consume thiosulfate. Washed cell suspensions will oxidize formate.

Thiobacillus novellus. Starkey's *T. novellus*, described in 1935 as a facultative autotroph (8), and the denitrifying thiobacillus of Tyulpanova-Mosevich (22) represent the exceptions to the rule of obligate autotrophy in the thiobacilli. In mineral media *T. novellus* closely resembled a nonmotile *T. thioparus*, but it grew profusely on organic substrates. Unfortunately subcultures of *T. novellus* have been maintained on organic media and at present oxidize thiosulfate only slowly. The "thiobacillus" of Trautwein (40), on the other hand, never carried out a very rapid thiosulfate oxidation and is probably more closely allied to some of the heterotrophic thiosulfate oxidizers described by Guittonneau (6), Starkey (8), and Sijderius (9). Trautwein's bacterium should therefore not be included in the genus *Thiobacillus*.

Other species. Several other isolates of *Thiobacillus* can justifiably be relegated to previously known species, as Starkey has already pointed out (8), the justification depending on the significance to be attached to cultural differences which various investigators have described. The unsatisfactory usage of the term "species" in bacteriology encourages the reviewers, like others before them, to be taxonomic lumpers. Issatchenko's *Thiobacterium beijerinckii* and *Thiobacterium nathansohnii* are probably strains of *Thiobacillus thioparus* (41). *Thiobacillus thermitanus*, *Thiobacillus lobatus*, *Thiobacillus umbonatus*, and *Thiobacillus crenatus*, described by Emoto and differentiated on the basis of colonial morphology, appear to belong to *Thiobacillus thiooxidans* (42, 43). *Thiobacillus concretivorus*, found by Parker to corrode concrete sewers in the presence of H_2S, likewise can be assigned to *Thiobacillus thiooxidans* (44). Thermophilic (45, 46), halophilic (47), and halotolerant (48) *Thiobacillus* strains have been found. A systematic search for various types of *Thiobacillus*, especially for facultative autotrophs, might well provide further support for Bunker's view that there is ". . . a gentle gradation of types from the strictly autotrophic organisms . . . through facultative autotrophs . . . to commoner types which may be little more than incidental oxidisers of sulphur and sulphur compounds" (49).

There remains one more organism which has been described as a thiobacillus. Lipman and McLees, in the course of attempts to isolate bacteria from a triassic coprolite, found an organism which slowly oxidized sulfur and thiosulfate and also grew slightly on organic media (50). It occurred as a very thin (0.1–0.2 μ) motile rod, vibrio, or spirillum, varying from 3 to over 40 μ in length, possessing a single polar flagellum. No gram stain was recorded. The authors concluded that the bacterium was a facultatively autotrophic *Thiobacillus*, and in 1940 designated it *Thiobacillus coproliticus*. No convincing evidence for the occurrence of viable bacteria in fossils has ever been presented (G. G. Simpson, private communication); but regardless of the origin of this organism, the reviewers favor its exclusion from the genus *Thiobacillus*. Growth on thiosulfate media appears to have been exceedingly poor to judge by the statement: "Thiosulfate broth: Good growth. No turbidity." Its singular morphology, its failure to acidify appreciably sulfur or thiosulfate-containing media, and its formation of "spores or regenerative bodies" differentiate the bacterium of Lipman and McLees sharply from the thiobacilli.

ISOLATION AND CULTIVATION OF THE THIOBACILLI

"Die Reinkultur sowohl der Süsswasser–wie der an den niederländischen Küsten ganz allgemeine Meeresform gelang auf ähnliche Weise, wie schon von Natanssohn angegeben worden ist."—M. W. Beijerinck (5).

The thiobacilli can be easily obtained by the enrichment culture technique from environments which are rich in reduced sulfur compounds. We have had excellent results using as inoculum marine mud gathered at low tide near a sewage disposal plant, although thiobacilli are also found in many other habitats. While these bacteria can be readily purified by streaking on solid media, cultures generally survive better in liquid media than on agar, possibly because high concentrations of sulfuric acid may arise in the immediate vicinity of a colony. The acid-sensitive *Thiobacillus thioparus*, for instance, requires transfer every three days, whether grown on liquid or on solid media. Thiobacillus cultures are therefore best preserved by lyophilization. The media described below contain ammonium salts, since only one *Thiobacillus* strain has been reported to use nitrate as a nitrogen source (44). Contrary to earlier impressions, even *T. denitrificans* requires ammonia for growth (21). Metal require-

ments of the thiobacilli were first demonstrated by Baudisch, who isolated *T. thiooxidans* from an ideal source: shallow pools encrusted with rhombic sulfur (51). *T. thioparus* requires iron and manganese (52), and *T. denitrificans* grows best with 0.25–8.3 μg Fe^{++} per ml (21).

Thiobacillus thioparus. Nathansohn isolated what is now recognized as *T. thioparus* from sea water (4), Beijerinck subsequently recovered the same organism from fresh water and the ever fruitful Delft canal mud (5), while Jacobsen (53, 54) and Starkey (8) demonstrated the presence of this bacterium in soils, muds, and waters of widely differing localities. Enrichment cultures of *T. thioparus* may be obtained by inoculating a shallow layer of the following medium: $Na_2S_2O_3 \cdot 5H_2O$, 10.0 g; KH_2PO_4, 4.0 g; K_2HPO_4, 4.0 g; $MgSO_4 \cdot 7H_2O$, 0.8 g; NH_4Cl, 0.4 g; trace metal solution[4], 10.0 ml; and H_2O, 1000 ml. In two or three days at 30 C the culture fluid becomes turbid and acid. A loopful is taken from the surface and streaked on an agar medium of the same composition. Within 48 hours small, opaque colonies appear, with an orange tint in the center of the larger ones. On the third day the colonies are white or yellow with precipitated sulfur. Two successive subcultures from single colonies assure bacteriological purity. In liquid media of the foregoing composition little or no sulfur is precipitated during growth, while a sulfur pellicle is formed on Starkey's (8) or Baalsrud's (21) medium. Pure cultures attain a density of 3×10^7 to 10^9 viable cells per ml in 24 to 48 hours.

For biochemical studies *T. thioparus* can be easily grown in the described medium. Carboys (12-gal capacity) are filled with 40 L of medium, which need not be sterilized, and fitted with two inlet tubes terminating in fritted glass candles. Air is blown through one candle as rapidly as practical, and CO_2 through the other at 5 to 10 per cent of the rate of air flow. As the pH begins to drop, the medium is constantly returned to pH 6.5 to 7.0 by the addition of solid Na_2CO_3. Once the addition of Na_2CO_3 has begun the flow of CO_2 can be cut off. Maximum growth is reached in about 60 hours at 30 C; the yield is usually 15 g wet weight per 40 L. These cells are virtually free of elemental sulfur and are very active in CO_2 fixation and in the oxidation of sulfur compounds.

Thiobacillus denitrificans. Although *T. denitrificans* is ubiquitous, it can be isolated most reliably from marine mud. A culture may be obtained by inoculating a glass stoppered bottle and filling it completely with the following medium (21): $Na_2S_2O_3 \cdot 5H_2O$, 5.0 g; KNO_3, 2.0 g; K_2HPO_4, 2.0 g; $NaHCO_3$, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.6 g; NH_4Cl, 0.5 g; $FeSO_4 \cdot 7H_2O$, 0.01 g; H_2O, 1000 ml. The final pH should be 7.0. K_2HPO_4, $NaHCO_3$, and $FeSO_4$ (stock solution in 0.1 N HCl) must be sterilized separately. We have also successfully cultured *T. denitrificans* with trace metal solution[4] (10 ml per liter) instead of $FeSO_4$. The bottles are incubated at 30 C until the medium turns turbid and gas bubbles rise from the sediment. The crude culture is then streaked on agar plates of the same composition and incubated in a desiccator anaerobically under a partial pressure of CO_2 of 50 mm Hg. Colonies of *T. denitrificans* are white or yellow with precipitated sulfur when Baalsrud's medium is used, but less visible sulfur is formed when the trace metal solution is employed. Pure cultures, which can be obtained by successive subculture of single colonies, can be maintained in agar stabs.

Thiobacillus thiooxidans. T. thiooxidans was originally isolated by Waksman and Joffe from soil enriched with elemental sulfur (24), but it may also be recovered from the same habitats as *T. thioparus.* Because of H_2S production by *Desulfovibrio aestuarii*, marine mud is the most reliable source of *T. thiooxidans.* It can be isolated from an enrichment culture using the same medium as is used for *T. thioparus*, except that the pH should be 3.5–4.0. In three to four days the pH may drop to 2.0, thereby virtually assuring the predominance of *T. thiooxidans*, although frequently a longer time is required. Pure cultures may be obtained by streaking on a solid medium of the same composition.

Thiobacillus ferrooxidans and Ferrobacillus ferrooxidans. The organism described as *T. ferrooxidans* has been isolated from acid mine water (26), and nothing is known about its distribution elsewhere. The enrichment medium used by Colmer *et al.* contains: $FeSO_4 \cdot 7H_2O$, 130 g (to make a final concentration of 2.6×10^4

[4] Trace metal solution: Ethylenediamine tetraacetic acid 50.0 g, $ZnSO_4 \cdot 7H_2O$ 22.0 g, $CaCl_2$ 5.54 g, $MnCl_2 \cdot 4H_2O$ 5.06 g, $FeSO_4 \cdot 7H_2O$ 4.99 g, $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ 1.10 g, $CuSO_4 \cdot 5H_2O$ 1.57 g, $CoCl_2 \cdot 6H_2O$ 1.61 g, H_2O 1000 ml. Adjusted to pH 6.0 with KOH.

ppm Fe^{++}); $MgSO_4 \cdot 7H_2O$, 1.0 g; $(NH_4)_2SO_4$, 0.5 g; H_2O, 1000 ml; and concentrated H_2SO_4 to make the pH 2.0 to 2.5. Presumably phosphate and trace metals are present as impurities. The organism was isolated on a solid medium of the same composition and can also be grown on a thiosulfate medium adjusted to pH 2.5. *Ferrobacillus ferrooxidans* has been cultivated by Leathen *et al.* on the following medium (30): $(NH_4)_2SO_4$, 0.15 g; KCl, 0.05 g; $MgSO_4 \cdot 7H_2O$, 0.50 g; K_2HPO_4, 0.05 g; $Ca(NO_3)_2$, 0.01 g; $FeSO_4 \cdot 7H_2O$, 1.0 g; and H_2O, 1000 ml, with a final pH of 3.5. The iron salt was added aseptically from a filter sterilized stock solution.

Thiobacillus thiocyanoxidans. T. thiocyanoxidans has been isolated from gas works liquor, sewage effluent, and thiocyanate-containing well water (38). It appears that this bacterium is sensitive to organic compounds and to some unidentified material present in commercial agar, although it is possible to select a population which will grow on washed agar. Silica gel has been used successfully in several isolations. Youatt employed the following medium (39): Na_2HPO_4, 1.0 g; KH_2PO_4, 0.6 g; KCNS, 0.2 g; $MgSO_4 \cdot 7H_2O$, 0.02 g; "a trace of iron" (*sic*); H_2O, 1000 ml. The final pH should be 7.0 to 7.2, and the bacterium was grown at 30 C. If thiosulfate is used as substrate, an ammonium salt and CO_2 must be provided.

Thiobacillus novellus. Starkey isolated *T. novellus* from one of a series of enrichment cultures which contained a medium suitable for *T. thioparus* and were inoculated with garden soil (8).

OXIDATION OF SULFUR COMPOUNDS

"In erster Linie fragt es sich, wie der Schwefeloxydationsprozess aufzufassen ist, von welchem das Leben der Schwefelbakterien abhängt. Es liegt sehr nahe zu vermuten, dass dieser Prozess hier der Atmung entspricht."—Sergei Winogradsky (2).

The oxidation of sulfide, sulfur, or thiosulfate to sulfate provides the energy for the growth of the thiobacilli. The intermediate compounds formed, and the relation of the oxidative steps to energy-storing and energy-utilizing reactions are still largely unknown.

Sulfide. Several other sulfur bacteria oxidize sulfide to sulfate with the intermediate formation of elemental sulfur. In the *Beggiatoaceae* (2) and *Thiorhodaceae* (55) sulfur accumulates visibly in the cells, while the *Chlorobacteriaceae* deposit sulfur outside the cells and frequently do not oxidize it further (55). *T. thioparus* rapidly oxidizes sulfide to sulfate, but no intermediate sulfur accumulation has been observed. Since there is no evidence for the intermediate formation of sulfur in the oxidation of sulfide, we must assume that either sulfur is not an intermediate, or subsequent transformations of sulfur are at least as rapid as the oxidation of sulfide to sulfur. Neither the deposition of sulfur in cultures of *T. thioparus* nor the transitory storage of sulfur in the vacuoles of *T. thiooxidans* (56) is to our knowledge comparable to the sulfur accumulation in the *Beggiatoaceae* and *Thiorhodaceae.*

The oxidation of sulfide by *T. thioparus* proceeds through polythionate intermediates. Resting cell suspensions of *T. thioparus* have been incubated with radiosulfide for varying lengths of time, extracted with 30 per cent ethanol, and the extract chromatographed on Dowex-1 with increasing concentrations of ammonium formate (unpublished observations). After one minute all radioactivity had disappeared from the sulfide peak and could be eluted with the thiosulfate and polythionate peak. On longer incubations the radioactivity of the combined thiosulfate and polythionate peak decreased and eventually could be recovered with eluted sulfate.

Sulfur. The oxidation of elemental sulfur is a distinguishing but not unique property of *T. thiooxidans.* Jacobsen has reported sulfur oxidation by *T. thioparus* (53), and so have Starkey (52) and Parker and Prisk (57). In a vigorously aerated culture of *T. thioparus*, growing on thiosulfate in our medium with constant neutralization, colloidal sulfur appears as a milky cloudiness in 36 hours (about 2 g per liter) but is completely oxidized to sulfate in six more hours. A similar observation has been made on *T. denitrificans* by Baalsrud and Baalsrud (21), who also found that resting cell suspensions will denitrify with colloidal sulfur as electron donor. Beijerinck (5) and Lieske (19) both grew *T. denitrificans* on elementary sulfur.

The mechanism of the bacterial attack on elemental sulfur is entirely unknown, although a number of ingenious hypotheses have been advanced. Vogler and Umbreit investigated the effect of surface active agents, particle size, and interposition of a dialyzing membrane on sulfur oxidation by *T. thiooxidans*, as well as the micro-

scopic behavior of these bacteria in the presence of sulfur, and concluded that direct contact between sulfur and the bacteria was required for oxidation to proceed (58). Upon contact the sulfur was thought to dissolve in a terminal fat globule and thus become susceptible to enzymatic oxidation in the bacterium (59). The requirement for physical contact implies that sulfur oxidation is favored in stagnant or slowly shaken flasks over rapidly shaking cultures (58), yet with *T. thioparus* we find rapid oxidation of colloidal sulfur during turbulent agitation as well as in standing cultures. Starkey *et al.* have reported an increase in the rate of sulfur oxidation by *T. thiooxidans* on vigorous shaking (59*a*), in agreement with Newburgh's observation (106). Electron micrographs of *T. thiooxidans* failed to confirm the presence of bipolar fat globules, but revealed instead a cell wall which would be an additional barrier (60). According to Knaysi the "fat globules" are vacuoles which ordinarily contain volutin, and immediately after sulfur oxidation both volutin and sulfur (56). It is this stored colloidal sulfur which may undergo chemical reactions similar to those of lipids [staining (56), iodine absorption (61)] and therefore give the impression of fat globules. In yeast volutin has been identified with cyclic (meta) and linear polyphosphates. The accumulation of polymetaphosphate in *T. thiooxidans* has been tentatively confirmed by Barker and Kornberg, who found large amounts of an easily hydrolyzable polyphosphate (62). Knaysi also observed a capsule which he thought should interfere with a close contact between sulfur and the bacteria (56), but the frequency with which such a capsule occurs seems to be in doubt (63).

Diffusion and reduction to sulfide have both been suggested as mechanisms for the transport of sulfur across the cell wall of *T. thiooxidans*. Diffusion is likely to be too slow in view of the infinitesimal solubility of sulfur in aqueous media, although it may account for processes which require only very small amounts of sulfur, such as fungicidal action (64). The possibility of sulfur reduction followed by diffusion or active transport of the sulfide into the cell has been discussed by Starkey who considers it unlikely, partly because he did not detect a rapid production of H_2S by *T. thiooxidans*, and partly because he believes that thermodynamic obstacles oppose it (65). Nevertheless, the common property of living cells to reduce sulfur may in *T. thiooxidans* have given rise to a specialized sulfur transport mechanism. Glutathione (reduced, GSH; oxidized, GSSG) or another sulfhydryl compound may serve as the functional group (66). The reaction

$$(1)\quad S^0 + 2\ GSH \rightarrow S^= + GSSG + 2\ H^+$$

yields sulfide which, free or in bound form, can enter the cell. Of the eight electrons transferred in the subsequent oxidation of sulfide to sulfate, two can be used to reduce GSSG to 2 GSH.

The oxidation of sulfur is likely to lead to the intermediate formation of thiosulfate and polythionates. Guittonneau and Keilling have found that elemental sulfur in soil disappears with the formation of thiosulfate, tetrathionate and other polythionates, and eventually sulfate (7), but these transformations are brought about by a mixed population of soil microorganisms. As one of several possibilities Fromageot mentions the condensation of sulfite and sulfur as the source of thiosulfate which arises during sulfur oxidation by mammals (67). We suggest that this reaction, enzymatically catalyzed, should be considered a possible step in the path of bacterial sulfur oxidation.

Thiosulfate. The first product of the bacterial oxidation of thiosulfate is tetrathionate. This oxidation occurs not only among the thiobacilli but also among the heterotrophic bacteria which oxidize thiosulfate. Gleen and Quastel showed that in soil perfused with a thiosulfate solution tetrathionate is formed, together with sulfur and sulfate. Soil, perfused with tetrathionate until the rate of tetrathionate oxidation is maximal, will oxidize thiosulfate without a lag (68). The occurrence of polythionates in the oxidation of thiosulfate by *T. thioparus* was first reported by Nathansohn, who found in growing cultures a sulfur compound which did not react with iodine but yielded sulfate on oxidation with bromine (4). Starkey's later attempts to demonstrate polythionate formation in thiobacillus cultures failed, most likely because enough time elapsed for all polythionates to be oxidized to sulfate before analyses were performed (69, 70). However, workers in several laboratories have confirmed Nathansohn's observation that polythionates, tetrathionate in particular, accumulate transiently during thiosulfate oxidation (21, 57, 71, 72).

The formation of intermediate oxidation products in a culture of *T. thioparus* becomes

TABLE 1

Partial oxidation of thiosulfate by Thiobacillus thioparus

	1	2
Thiosulfate consumed (μmoles)...	9.6	9.4
Alkali formed* (μequivalents)....	9.7	9.5
O_2 consumed (μmoles).....	2.3	2.3

* Determined as increase in bicarbonate.

Each vessel contained bacterial matter equivalent to 1.0 mg nitrogen, and 20 μmoles of $NaHCO_3$. Volume, 2.0 ml. Atmosphere, air with 10 per cent CO_2. Temperature, 30 C. Experiment 1 was conducted with unwashed dried cells, experiment 2 with a stored suspension of intact cells. No detectable thiosulfate remained at the end of the experiment. The presence of tetrathionate was indicated by a yellow precipitate formed upon addition of $HgNO_3$.

obvious from a measurement of growth and thiosulfate utilization: only one third of final growth (measured as organic N) has been attained at the time all detectable thiosulfate has been consumed (72). The evidence for tetrathionate is based on chemical and manometric determinations. A resting suspension of cells grown on Starkey's medium[5] will oxidize thiosulfate at a rate which changes sharply when 0.25 μmole O_2 per μmole thiosulfate have been consumed. Stored suspensions of such cells and certain preparations of dried cells will frequently limit their total oxygen uptake to 0.25 μmole per μmole thiosulfate. The results of two such experiments are given in table 1, and can best be accounted for by the reaction

$$(2)\ S_2O_3^{=} + 0.25\,O_2 + 0.5\,H_2O \rightarrow 0.5\,S_4O_6^{=} + OH^-$$

Baalsrud and Baalsrud have made similar observations on *T. denitrificans* (21), and have pointed out, furthermore, that the observation of the telltale break in the rate of thiosulfate oxidation was a fortuitous accident brought about by injury which the culture suffered from the rise in acidity during growth. In agreement with their view we have found that *T. thioparus* grown in our present medium with continuous neutralization will invariably oxidize thiosulfate at a single linear rate until oxidation is complete. A rapid and quantitative oxidation of thiosulfate to sulfate has also been achieved with dried cells of *T. thioparus*.

[5] $Na_2S_2O_3 \cdot 5H_2O$ 10.0 g, K_2HPO_4 4.0 g, KH_2PO_4 4.0 g, $CaCl_2$ 0.10 g, $MgSO_4 \cdot 7H_2O$ 0.10 g, $(NH_4)_2SO_4$ 0.10 g, $FeCl_3 \cdot 6H_2O$ 0.02 g, $MnSO_4 \cdot 2H_2O$ 0.02 g, H_2O 1000 ml (46).

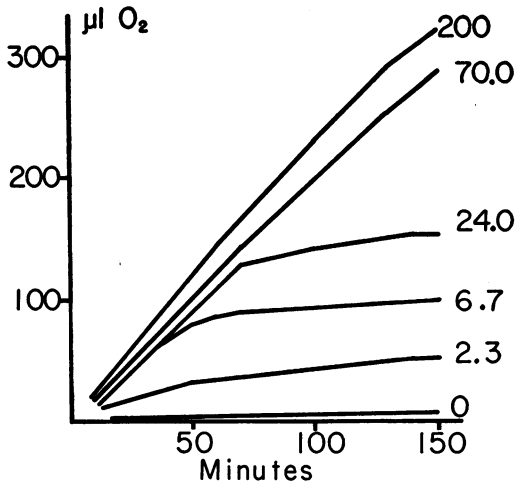

Figure 1. Effect of phosphate on the oxidation of tetrathionate by *Thiobacillus thioparus*. Each flask contained 8 mg dry weight of bacteria, 5 μmoles of $S_4O_6^{=}$, and 1.0 mmole of KOH in the center well. Phosphate buffer, pH 7.0, was added to the vessels in the indicated quantities (in μmoles).

Tetrathionate. Tetrathionate is oxidized rapidly by *T. thioparus* (both our own and Starkey's strain), *T. thiooxidans* (Starkey's strain) (72), *T. denitrificans* (21), and by the green sulfur bacterium *Chlorobium thiosulfatophilum* (73). We have found that tetrathionate oxidation can be observed most readily with freshly harvested cells, using sodium tetrathionate which has been recrystallized several times, in the presence of an adequate amount of phosphate. The effect of phosphate concentration on tetrathionate oxidation, shown in figure 1, is specific and independent of its buffering capacity. The oxidation of thiosulfate beyond the tetrathionate stage is similarly phosphate dependent (see *Energy Metabolism and Assimilation*). Parker and Prisk (57) report that their *Thiobacillus* X (apparently identical with our *T. thioparus*) oxidized tetrathionate, but failed to find tetrathionate oxidation with Starkey's strain of *T. thioparus* and with two strains of *T. thiooxidans* (Starkey's strain and their *T. concretivorus*). Tamiya *et al.* concluded that their culture of *T. thioparus* (apparently identical with our own) could not

oxidize tetrathionate (70), but we believe that they may have worked with virtually inactive suspensions. Their bacteria were grown without added Fe^{++} and Mn^{++}, attaining a density of only 0.8 to 2.5 mg dry weight per liter (as compared to our 50 mg dry weight per liter), were unable to oxidize thiosulfate to completion and did not evolve CO_2 during endogenous respiration.

The fate of tetrathionate is obscure, but there is evidence that trithionate or a closely related compound is formed during tetrathionate oxidation. Gleen and Quastel noted that soil enriched with sulfur-oxidizing bacteria by perfusion with thiosulfate will, at low concentrations, oxidize tetrathionate and trithionate without a lag and at the same rate as thiosulfate (68). On the other hand, *Chlorobium thiosulfatophilum* which oxidizes thiosulfate and tetrathionate failed to oxidize trithionate (73). Tamiya *et al.* (71, 74) and we (72) have observed trithionate oxidation by *T. thioparus*. In thiosulfate oxidation by *T. thioparus* the rate of oxygen uptake may undergo a sudden change at a point which corresponds to the oxygen uptake required to convert thiosulfate to trithionate (0.67 μmole O_2 per μmole $S_2O_3^=$). An equivalent break in the rate of tetrathionate oxidation also occurs (0.83 μmole O_2 per μmole $S_4O_6^=$). As was mentioned previously such changes cannot be observed under our present, more favorable, conditions. Finally, a transient accumulation of trithionate has been demonstrated (72). Tetrathionate may therefore undergo oxidations which, at least in part, give rise to trithionate. The manometric data, which alone, of course, are not conclusive, are in agreement with a quantitative conversion of tetrathionate to trithionate

$$(3)\quad 3\ S_4O_6^= + 2.5\ O_2 + H_2O \rightarrow 4\ S_3O_6^= + 2\ H^+$$

Attempts to unravel such a reaction sequence in terms of individual enzymatic steps should be aided considerably by the studies of Foss on the structure and properties of polythionates (75).

The oxidation products of trithionate, other than the ultimate sulfate, are unknown. No evidence exists for the formation or utilization of sulfite. Dithionate is only slowly oxidized by *T. thioparus* (52, 72), and soil perfusion experiments indicate no relationship between thiosulfate and dithionate oxidation (68).

Sulfur formation. Nathansohn observed that a culture medium, from which *T. thioparus* had been filtered after a short period of growth, would become turbid with colloidal sulfur (4), and that this formation of sulfur in the absence of bacteria could be inhibited with cyanide. These experiments have at times been misinterpreted in the sense that a cyanide sensitive extracellular enzyme was responsible for sulfur deposition, although Nathansohn himself expressed a different view: "Die qualitative und quantitative Untersuchung der Stoffwechselproducte brachte eine Aufklärung über diesen Punkt, indem sich zeigte, dass die extracelluläre Schwefelausscheidung einem secundären Processe entspringt, intracellulär aber die Oxydation des Thiosulfates in ganz anderer Weise vor sich geht." According to Starkey sulfur arises by a specific biological reaction which is part of the mechanism of thiosulfate oxidation. His analyses led him to the formulation (70):

$$(4)\quad 5\ S_2O_3^= + 4\ O_2 + H_2O \rightarrow 6\ SO_4^= + 4\ S^0 + 2\ H^+$$

Tamiya *et al.* expressed the course of thiosulfate oxidation as the sum of five reactions (71):

$$(5)\quad S_2O_3^= + 0.25\ O_2 + 0.5\ H_2O \rightarrow 0.5\ S_4O_6^= + OH^-$$

$$(6)\quad 2\ S_4O_6^= \rightarrow S_3O_6^= + S_5O_6^=$$

$$(7)\quad S_5O_6^= \rightarrow S_4O_6^= + S^0$$

$$(8)\quad S_3O_6^= + H_2O \rightarrow S_2O_3^= + SO_4^= + 2\ H^+$$

$$(9)\quad S_3O_6^= + 2\ O_2 + 2\ H_2O \rightarrow 3\ SO_4^= + 4\ H^+$$

Only reactions 5 and 9, the oxidation of thiosulfate and trithionate, were thought to be enzymatic. Trithionate, therefore, of necessity arises in this scheme as one product of a nonbiological dismutation of tetrathionate, reaction 6, since according to these authors *T. thioparus* does not attack tetrathionate. A nonbiological decomposition, reaction 7, gives rise to sulfur, and a nonbiological hydrolysis of trithionate, reaction 8, to some of the sulfate. Three objections to this scheme can be raised. First, *T. thioparus* is able to oxidize tetrathionate rapidly. Second, thiosulfate can be oxidized to completion, without any sulfur production, by *T. thioparus*, even by dried cells which will not oxidize sulfur once it is formed, although sulfur production is obligatory in both Starkey's and Tamiya's schemes. Finally, reactions 6 and 7, which here form an essential link in the chain of oxida-

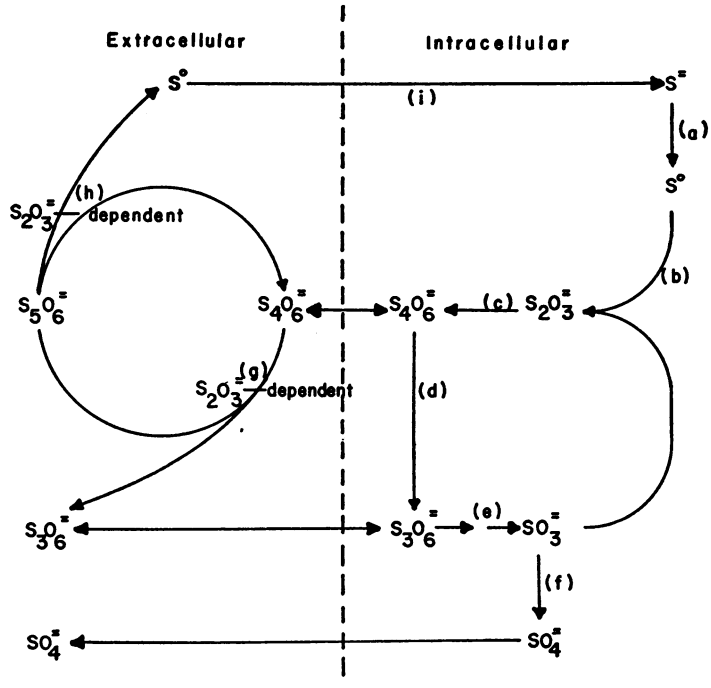

Figure 2. Postulated path of sulfur oxidation. For explanation see text.

tive events, are too slow to account for the growth of *T. thioparus* (76), even though they are greatly accelerated by thiosulfate (75). The stability of tetrathionate is adequately demonstrated by Tamiya's own data (figure 2 in reference 71), which show a constant tetrathionate level, once thiosulfate has disappeared, for 11 days in a culture of *T. thioparus*.

Tamiya's suggestion that the sulfur precipitated in *T. thioparus* cultures originates by reactions 6 and 7 is supported by several observations, with these differences: (a) the reactions are not an integral part of the path of thiosulfate oxidation, but instead are a wasteful side reaction by which substrate is lost; and (b) reactions 6 and 7 are dependent on thiosulfate as a catalyst. In cultures containing initially 1 per cent sodium thiosulfate both thiosulfate and tetrathionate are present simultaneously for up to two days, hence sulfur is formed. Saslawsky (47) and Lange-Posdeeva (48) found that on lower thiosulfate concentrations no sulfur was precipitated by their strains of *T. thioparus*. In manometric experiments no sulfur is precipitated because all thiosulfate is converted to tetrathionate in a matter of minutes, unless very large amounts of thiosulfate are used (72). Nathansohn's experience in inhibiting sulfur formation with KCN (4) can be understood, since KCN reacts with the polythionates present and therefore removes the precursors of sulfur:

(10) $S_4O_6^- + CN^- + H_2O \rightarrow S_2O_3^- + SCN^- + SO_4^- + 2\ H^+$

(11) $S_5O_6^- + 2\ CN^- + H_2O \rightarrow S_2O_3^- + 2\ SCN^- + SO_4^- + 2\ H^+$

Path of thiosulfate oxidation. The results and speculations discussed in the preceding sections have led us to the scheme outlined in figure 2 as an admittedly incomplete and speculative working hypothesis. Not all the intermediates shown here need exist as free compounds in the cell; we believe that one or more phosphorylated compounds are formed.

In this scheme sulfide enters the cell and is oxidized to sulfur (reaction *a*). Sulfur in the cell undergoes an enzymatic condensation with sulfite (reaction *b*) to form thiosulfate which is then oxidized to tetrathionate (reaction *c*). The oxidation of tetrathionate gives rise to trithionate (reaction *d*) and perhaps sulfite (reaction *e*). Sulfite is oxidized to sulfate (reaction *f*), but some of it may be recycled through

reaction *b*. Should *Thiobacillus* be oxidizing thiosulfate, instead of sulfide, and thiosulfate and tetrathionate be present simultaneously for several hours, then some tetrathionate will dismute to give rise to trithionate and pentathionate (reaction *g*), and the latter by another thiosulfate catalyzed reaction will produce sulfur and tetrathionate (reaction *h*). Reaction *a* is known to occur in the *Beggiatoaceae* and in the photosynthetic sulfur bacteria. Reactions *c* and *d* have been observed in *T. thioparus*. Reactions *g* and *h* are known inorganic reactions. Reactions *b*, *e*, and *f* are hypothetical. Reaction *i* represents the possible utilization of sulfur by reaction with sulfhydryl groups.

Denitrification. T. denitrificans will oxidize sulfur compounds anaerobically with nitrate as terminal electron acceptor, although it can also use oxygen as do other thiobacilli. A discussion of denitrification is beyond the scope of this review; the reader is therefore referred to the investigations on *T. denitrificans* by Baalsrud and Baalsrud (21), who were the first to observe the formation of NO in denitrification, and to other summaries of our current knowledge of this process (77).

CARBON AND ENERGY METABOLISM

"Die Thatsache der chemosynthetischen Kohlensäure-Assimilation lehrt, wie mir scheint... dass die bei der Oxydation frei werdende Energie wenigstens zum Theil nicht als Wärme auftritt, sondern sofort transformirt und dazu verwendet wird, Körper von hohem chemischem Energiepotential zu schaffen."—Alexander Nathansohn (4).

Metabolism of organic compounds. The inability of the obligately autotrophic thiobacilli to utilize external organic substrates has prevented a study of their organic metabolism by the simple expedient of incubating cell suspensions with a variety of organic compounds. It is clear, nevertheless, that aside from the oxidation of sulfur their internal physiology is essentially similar to that of many other microorganisms. Vogler observed an endogenous respiration (R.Q. = 1) in *T. thiooxidans* (78), and concluded that an organic storage product which had been synthesized from CO_2 during sulfur oxidation was being oxidized. The storage material, according to LePage, is a polysaccharide consisting mostly of glucose and mannose (79). Other analytical data confirm that the thiobacilli contain the usual cell constituents, including at least six of the B vitamins (80), several hexose phosphates (81), and coenzymes (81). The occurrence of other common cell components is evident from the fact that we have repeatedly grown batches of *T. thioparus* on $C^{14}O_2$ in order to furnish several investigators with totally labeled amino acids, purines, pyrimidines, and nucleotides. Frantz *et al.* have obtained totally labeled amino acids from *T. thiooxidans* (82). The claim of LePage and Umbreit that *T. thiooxidans* contains a specific ATP, adenosine-3′-triphosphate (83), proved to be erroneous; only adenosine-5′-triphosphate was found by Barker and Kornberg (62).

Although externally supplied organic compounds do not support growth of the obligately autotrophic thiobacilli, at least some such compounds penetrate into the cell. Waksman and Starkey (84) and Emoto (85) found that the rate of sulfur oxidation by *T. thiooxidans* increased slightly in the presence of glucose. The glucose present during sulfur oxidation slowly disappeared, and the amount of glucose consumed was proportional to the growth of the organism (17). In their manometric studies Vogler *et al.* noted that the C_4-dicarboxylic acids enhanced the rate of respiration of *T. thiooxidans* (15). *T. thioparus* not only responds to the C_4-dicarboxylic acids with an increased oxygen uptake, but, as is shown in table 2, its growth on Starkey's medium[5] is increased 20 to 40 per cent in the presence of these acids (16). This increase is independent of the concentration of the added acids over the range 10^{-3}–10^{-1} M; furthermore, the added acids have no effect in the absence of either CO_2 or thiosulfate. Since glucose is consumed by *T. thiooxidans* and succinate stimulates the respiration of *T. thiooxidans* and *T. thioparus*, these compounds must be able to enter the cell. Obligate autotrophy cannot therefore be explained in terms of cell walls or membranes impermeable to organic matter. The increased growth of *T. thioparus* in the presence of succinate may be a consequence of the respiratory stimulation. The amount of growth on Starkey's medium in the absence of organic compounds is a function of the amount of thiosulfate oxidized to sulfate, or total sulfur minus precipitated elemental sulfur. From the discussion of sulfur formation it follows that slow growth will result in low bacterial yields since there will be time for considerable substrate to be lost by sulfur formation, while rapid

TABLE 2

Growth of Thiobacillus thioparus in the presence of C_4-dicarboxylic acids

Added Acid Final Concentration: 10^{-3} M	Bacterial N
	mg
None	0.773
Succinate	1.09
Fumarate	1.11
Malate	1.12

The bacteria were grown in 100 ml of Starkey's medium[5] at 30 C for six days in a stream of air +5 per cent CO_2.

growth will end with a higher bacterial yield. As expected, growth on our present medium, on which no sulfur precipitation occurs, is not improved by succinate.

Substrate level phosphorylation. The rate and extent of the oxidation of thiosulfate to sulfate is dependent on the phosphate concentration in the medium, just as tetrathionate oxidation is phosphate dependent (figure 1). Tris[hydroxymethyl] aminomethane buffer (Tris) will replace phosphate only in part; a specific requirement for phosphate remains. *T. thioparus* cells suspended in Tris buffer will oxidize thiosulfate or tetrathionate to some stage short of sulfate, as judged by the cessation of oxygen uptake before the stoichiometric amount has been consumed. No detectable thiosulfate remains at this point. Upon the addition of phosphate, oxygen consumption resumes immediately and continues until the expected amount has been taken up (figure 3). Arsenate replaces phosphate completely. One or more steps in thiosulfate oxidation are therefore phosphate dependent reactions leading possibly to a mixed anhydride of the type —S—O—$PO_3^=$. Such a compound may in a subsequent reaction step donate phosphate to ADP to make ATP. Chromatographic analysis of a reaction mixture in which *T. thioparus* was supplied with S^{35} and $P^{32}O_4^=$ indicated the presence of one or more compounds containing both S^{35} and P^{32}. The formation of a phosphoryl sulfate (adenosyl-3′-phosphate-5′-phosphoryl sulfate) in several tissues has been described (86–88).

Electron transport phosphorylation and phosphate storage. During the oxidation of sulfur compounds by *Thiobacillus thiooxidans*, *T. thioparus*, and *T. denitrificans* a small but consistent shift of phosphate from orthophosphate to phosphate esters can be observed (89–91). We have observed a rapid incorporation of $P^{32}O_4^=$ into ATP during thiosulfate oxidation by intact cells of *T. thioparus*. Presumably the increased rate of phosphorylation during oxidation results in a

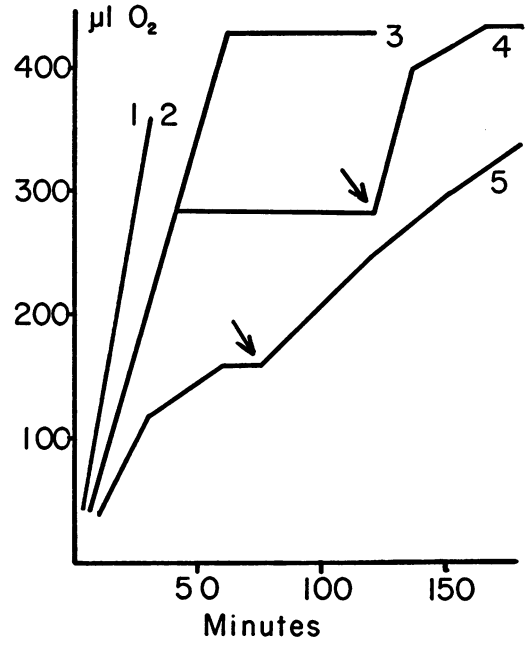

Figure 3. Effect of phosphate and arsenate on the oxidation of thiosulfate by *Thiobacillus thioparus*. *Experiment 1* (curves 1 and 2): A comparison of phosphate and arsenate buffers. Each flask contained 3.3 mg dry weight of bacteria, 8.4 µmoles of $S_2O_3^=$, and 1.5 mmole KOH in the center well. Flask 1 contained 75 µmoles phosphate buffer, pH 7.2, in a total volume of 2.0 ml; flask 2 contained 100 µmoles arsenate buffer, pH 7.2, in a total volume of 2.5 ml. *Experiment 2* (curves 3 and 4): Oxidation of $S_2O_3^=$ in Tris buffer, followed by addition of phosphate. Each flask contained 3.0 mg dry weight of bacteria, 10 µmoles of $S_2O_3^=$, and 1.5 mmole KOH in the center well. Flask 3 contained 200 µmoles phosphate buffer, pH 7.2, in a total volume of 2.8 ml, at zero time; flask 4 initially contained 100 µmoles Tris buffer, pH 7.2, and 100 µmoles phosphate buffer, pH 7.2, was added at 120 minutes (arrow) to make a final total volume of 3.0 ml. *Experiment 3* (curve 5): Oxidation of $S_2O_3^=$ in Tris buffer, followed by addition of arsenate. The flask contained 2.6 mg dry weight of bacteria, 8.4 µmoles of $S_2O_3^=$ and 1.5 mmole of KOH in the center well. Initially 150 µmoles of Tris buffer, pH 7.2, were present; at 75 minutes (arrow) 50 µmoles of arsenate buffer, pH 7.2, were added. Final volume, 2.5 ml.

higher steady state concentration of phosphate esters. Some of this phosphorylation may be assumed to occur during electron transport, although no pertinent experiments have been carried out with thiobacilli. Perhaps evidence indicative of electron transport phosphorylation can be sought in the observation of Vogler *et al.* that 2,4-dinitrophenol at low concentrations stimulates the respiration of *T. thiooxidans* (15).

The observation of Knaysi (56) that volutin (polymetaphosphate?) is formed in *T. thiooxidans* during substrate oxidation is consistent with the suggestion that polymetaphosphate could serve as an energy reservoir (92). Evidence for this suggestion is provided by experiments on P^{32} exchange between polymetaphosphate and ATP in yeast (93) and on the utilization of polymetaphosphate in the phosphorylation of glycerol in *Mycobacterium smegmatis* (94). In thiobacilli the accumulated polymetaphosphate may therefore represent an energy stockpile which is accessible through the reaction

(12) $ATP^{\equiv} + (PO_3^-)_n \rightleftharpoons ADP^{=} + (PO_3^-)_{n+1}$

Carbon dioxide fixation. Photosynthetic and chemosynthetic organisms both utilize CO_2 as the carbon source for the formation of all cell products. The elucidation of the path of carbon in photosynthesis by the radiocarbon studies of Calvin and his collaborators and the enzyme studies of Horecker and Racker (reviewed in 95), suggested the occurrence of similar mechanisms in the autotrophic bacteria. Crude extracts of *T. thioparus* will fix CO_2 in the presence of ribulose-1,5-diphosphate with the formation of 3-phosphoglyceric acid (96, 97); the radioactivity of the $C^{14}O_2$ can be recovered almost entirely from the carboxyl carbon. Intact cells of *T. thioparus* will rapidly incorporate $C^{14}O_2$ into 3-phosphoglyceric acid and sugar phosphates. The presence of the carboxylating enzyme was also established by an enzymatic assay based on the subsequent reduction of 3-phosphoglyceric acid to 3-phosphoglyceraldehyde in the presence of adenosine triphosphate, 3 - phosphoglyceric kinase, reduced diphosphopyridine nucleotide, and triosephosphate dehydrogenase. Ribose-5-phosphate isomerase and ribulose-5-phosphate kinase have also been found.

In extracts of *T. denitrificans* Trudinger has found that upon the addition of ATP and ribose-5-phosphate $C^{14}O_2$ is fixed in the carboxyl group of 3-phosphoglyceric acid, indicating the presence of ribose-5-phosphate isomerase, ribulose-5-phosphate kinase, and the carboxylating enzyme (98). The presence of glucose-6-phosphate dehydrogenase, 6-phosphogluconic dehydrogenase, enolase, aldolase, glucose-6-phosphate isomerase, fructose-1,6-diphosphatase, transaldolase, transketolase, triosephosphate dehydrogenase, 3-phosphoglyceric kinase, and phosphoglyceromutase was also established (99). More recently Aubert *et al.* (100) and Milhaud *et al.* (101) have shown that upon the addition of $C^{14}O_2$ to cells of *T. denitrificans* the distribution of radioactivity among the phosphorylated intermediates is similar to that obtained by Bassham *et al.* with *Chlorella* (102). Although Milhaud *et al.* did not examine the amount of C^{14} incorporated in compounds related to the tricarboxylic acid cycle, it appears reasonable that CO_2 fixation in *T. denitrificans* follows the same path as in photosynthetic organisms.

We have examined several hydrogen oxidizing bacteria (*Hydrogenomonas* spp.) for comparative purposes, and have found that here, too, CO_2 can be fixed by the carboxylation of ribulose-1,5-diphosphate. These bacteria are facultative autotrophs, and the carboxylating enzyme is formed adaptively under autotrophic conditions. When various strains are grown on lactate the carboxylating activity is reduced to a low level. It should be pointed out that this enzyme is not restricted to autotrophic organisms, but that *Escherichia coli* grown on a pentose in the presence of CO_2 will make 3-phosphoglyceric acid from ribulose-1,5-diphosphate and CO_2 (103).

Energy storage and CO_2 fixation. Vogler and Umbreit performed experiments on sulfur oxidation and CO_2 fixation in *T. thiooxidans* which led them to the following conclusions (89, 104): (a) In the absence of CO_2 sulfur is oxidized with a concomitant esterification of orthophosphate. (b) In the absence of sulfur, cells which have previously oxidized sulfur will fix CO_2 to an extent which is a function of the phosphate esterified during the preceding sulfur oxidation. (c) The esterified phosphate is again converted to orthophosphate during CO_2 fixation. These conclusions may be illustrated by the results of one experiment (89): a bacterial suspension (0.168 mg N per ml) in phosphate buffer was brought in contact with sulfur. In six hours 1650 μL O_2 and 3.9 μg P were taken up per ml in the absence of CO_2. The suspension was then flushed with H_2 to prevent further sulfur oxidation and

CO_2 was admitted in the absence of O_2; 125 μL CO_2 were then fixed per ml of suspension.

According to these authors the phosphate esterification during sulfur oxidation reflects an energy storage which subsequently enables the cell to fix CO_2 without simultaneous substrate oxidation. This interpretation is difficult to accept on the basis of the figures just cited. Bacteria equivalent to 0.168 mg N (corresponding roughly to 1.68 mg dry weight) fix 125 μL (5.58 μmoles) CO_2. As CO_2 acceptors we may consider here ribulose diphosphate or phosphoenolpyruvate; since other possible CO_2 acceptors are not likely to have substantially smaller molecular weights, the assumption of such other acceptors will not affect the argument. The fixation of 5.58 μmoles CO_2 then requires either 1.74 mg ribulose diphosphate or 0.94 mg phosphoenolpyruvate. These amounts are clearly incompatible with the amount of bacterial matter available.

An alternative interpretation is that CO_2 acceptors are present only in catalytic amounts, and that they are regenerated by reduction and recycling of the primary fixation products. The reduction of CO_2 to the carbohydrate level requires 4 H or, in terms of a reduced coenzyme, 7.58 mg DPNH—about five times the total dry weight of bacterial matter. It is possible that the sulfur remaining in suspension or in the bacteria when the atmosphere is changed from O_2 to H_2 serves as H-donor and that the necessary phosphate is stored as polymetaphosphate. On the basis of photosynthetic CO_2 fixation (reaction 13), polymetaphosphate utilization (reaction 12), and the as yet unfounded assumption that every oxidative step from S^0 to $SO_4^=$ is DPN linked (reaction 14), 1.34 mg of polymetaphosphate and 0.119 mg S^0 are required. This amount of polymetaphosphate is unlikely to be stored in 1.68 mg of bacteria, and Umbreit, in a reexamination of this experiment, found no $SO_4^=$ released during CO_2 fixation (105).

$$(13)\quad 3\ CO_2 + 9\ ATP^{\equiv} + 6\ TPNH\ (\text{or DPNH}) + 5H_2O \rightarrow \text{glyceraldehyde-3-P}^- + 8PO_4^= + 6\ TPN^+\ (\text{or } DPN^+) + 11\ H^+$$

$$(14)\quad S^0 + 3\ DPN^+ + 4\ H_2O \rightarrow SO_4^= + 3\ DPNH + 5\ H^+$$

Attempts by other investigators to repeat the observations of Vogler and Umbreit have failed. Baalsrud and Baalsrud studied the relationship between phosphorylation and CO_2 fixation in *T. thiooxidans*, *T. thioparus*, and *T. denitrificans* (90). They confirmed the uptake of small amounts of orthophosphate during substrate oxidation, but despite many experiments under a variety of conditions were unable to detect CO_2 fixation separated in time from substrate oxidation. Umbreit more recently has repeated his experiments, using $P^{32}O_4^=$ and $C^{14}O_2$ to follow phosphorylation and CO_2 fixation (105). The data on phosphorylation were refined by a distinction between orthophosphate in the medium and orthophosphate in the cell. Umbreit concluded that the most significant change during sulfur oxidation by *T. thiooxidans* is the shift from orthophosphate to ester phosphate inside the cell, which is inadequately reflected by uptake of orthophosphate from the medium. He found, in agreement with his earlier work, that CO_2 is fixed by cells which an hour previously have oxidized sulfur, and that this fixation is a function of the O_2 uptake during the sulfur oxidation. The CO_2 uptake is calculated from radioactivity incorporated in the course of one hour and is probably too high, since a certain amount of exchange is bound to occur.

Radioactive CO_2 and $PO_4^=$ have also been used by Newburgh in an attempt to check Vogler and Umbreit's results (106). In agreement with the other investigators he detected small changes in the orthophosphate level, although pronounced phosphorylation occurred only in the presence of O_2, CO_2, and sulfur. $C^{14}O_2$ assimilation was measured in *T. thiooxidans* cells after a variety of treatments: aerobic and anaerobic preincubation, with and without CO_2, in the presence and absence of sulfur. Small amounts of CO_2 were taken up in all instances, regardless of whether sulfur oxidation had preceded exposure to CO_2 or not. In the absence of O_2 no CO_2 fixation was found of the magnitude reported by Vogler and Umbreit; only in the presence of both O_2 and sulfur was there a vigorous uptake of CO_2. It is important that Newburgh observed a considerable loss of the ability to fix CO_2 in *T. thiooxidans* cells preincubated without CO_2, since these were the conditions under which Vogler and Umbreit observed storage of carboxylating ability. Aubert *et al.* (100) and Milhaud *et al.* (101) found a small but detectable fixation of $C^{14}O_2$ by *Thiobacillus denitrificans* in the absence of thiosulfate and nitrate. The amount of CO_2 fixed was about 1 per cent of the amount fixed in the presence of

thiosulfate and nitrate in one minute, and did not increase after that time. This fixation, which went to completion in less than one minute, took place at the expense of the ribulose-1,5-diphosphate which was present in the cells, while 3-phosphoglyceric acid accumulated. It is unlikely therefore, that orthophosphate was liberated as a consequence of this CO_2 fixation.

What information can we extract from this controversy? There seems to be general agreement that a small phosphate uptake can be observed during substrate oxidation, but the claim of a definite relationship between phosphorylation and subsequent CO_2-fixation by whole cells has not been substantiated. As regards their conclusions, the contention of Vogler and Umbreit that phosphate bond energy is required for CO_2 fixation is not only reasonable but has been borne out by the knowledge of CO_2 fixation which has accumulated since their original experiments were performed. It is also obvious that a CO_2 fixation of a magnitude reported by these authors requires the storage of an equivalent reducing power in addition to stored phosphate bond energy, but this point has not received sufficient attention. Whether or not CO_2 fixation in whole cells can be separated in time from phosphorylation has today little bearing on the study of phosphate bond utilization in CO_2 fixation. The catalytic nature of the CO_2 fixing and phosphate bond generating processes makes it unlikely that any sizable CO_2 fixation can occur unless all participating reactions, substrate oxidation, oxidative phosphorylation, and CO_2 fixation take place concurrently. These considerations and the experiences of the Baalsruds (90), Newburgh (106), Aubert *et al.* (100), and Milhaud *et al.* (101) cast doubt on the occurrence of a postoxidative CO_2 fixation of the magnitude reported by Vogler and Umbreit.

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